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Combining nucleoside analogues to achieve recognition of oligopurine tracts by triplex-forming oligonucleotides at physiological pH

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Abstract **We have used DNase I footprinting to examine DNA triple helix formation at a 12 base pair oligopurine · oligopyrimidine sequence, using oligonucleotides that contain combinations of 2′-aminoethoxy-5-(3-aminoprop-1-ynyl)uridine (bis-amino-U, BAU) and 3-methyl-2-aminopyridine (^{Me}P) in place of T and C, respectively. This combination acts cooperatively to enable high affinity triple helix formation at physiological pH. The affinity depends on the number of substitutions and their arrangement; oligonucleotides in which these analogues are evenly distributed throughout the third strand bind much better than those in which they are clustered together.**

Keywords: Triple helix; Nucleoside analogue; Footprinting; DNA sequence recognition

1. Introduction

Triplex-forming oligonucleotides (TFOs) bind specifically within the major groove of double-stranded DNA, forming a three-stranded structure [1]. Their unique base recognition properties make them ideal as gene-targeting agents for exploitation in medicine and biotechnology [2–5]. TFOs composed of pyrimidine bases bind in a parallel orientation to the purine strand of an oligopurine · oligopyrimidine duplex, forming T · AT and C^+ · GC triplets [6]. Their use is currently limited by their weak binding affinity and the requirement for conditions of low pH (pH < 6.0), necessary for protonation of the third strand cytosines. There has been considerable interest in the synthesis of modified TFOs designed to overcome each of these restrictions [3,7].

The affinity of TFOs for their duplex targets is generally low, as a consequence of charge repulsion between the three polyanionic strands. This can be overcome by using high concentrations of monovalent cations or low concentrations of divalent metal cations [6,8,9]. Studies on TFOs that contain natural bases have shown that protonated C has a much higher affinity for GC than T has for AT, attributed to the presence of the positive charge or improved base stacking interactions [10–14]. This led to the synthesis of modified TFOs that contain positive charges attached to either the base and/or sugar [15–22], or within the phosphate backbone [23]. 2′-Aminoethoxy-5-(3-aminoprop-1-ynyl)uridine (BAU), shown in Fig. 1A, is the most useful nucleotide analogue in this respect for recognition of AT base pairs. A 5-propargylamino group on the base and an aminoethoxy group on the 2′-position of the sugar place positively charged groups close to phosphate residues within the TFO and the duplex purine strand [21,22]. This nucleoside analogue dramatically increases TFO affinity without decreasing selectivity and it also removes the requirement for divalent metal ions.

The pH dependence of parallel triplex formation has also been addressed using oligonucleotides containing cytosine analogues. The pK_a of free cytosine is 4.5, though this may be elevated within a triple helix, depending on its position and sequence context [10–12]. Several cytosine analogues with high pK_a values have been synthesized [24–28] as well as pH independent cytosine mimics [29–33]; some of the most successful are those that retain the positive charge, such as 2-aminopyridine (P) and its 3′-methyl derivative (^{Me}P) which have pK_a values that are two pH units higher than cytosine [25–28] and are shown in Fig. 1B.

BAU and ^{Me}P, when used separately, have been shown to enhance triplex affinity and to extend the useful pH range for triplex formation, respectively. It is known that the base composition of an unmodified parallel TFO affects its affinity for its duplex target [13,14]. We have therefore examined the affinity and pH dependence of TFOs containing different numbers and arrangements of these nucleotide analogues when used in combination. These are compared to TFOs containing, 5-propargylamino dU (U^P) and 2-aminopyridine (P) as well as the natural bases T and C.

2. Materials and methods

2.1. Oligonucleotides

All oligonucleotides were synthesized on an Applied Biosystems ABI 394 automated DNA/RNA synthesizer on the 0.2 or 1 μmol scale using the standard cycles of acid-catalysed detritylation, coupling, capping and iodine oxidation procedures. Phosphoramidite monomers and other reagents were purchased from Applied Biosystems or Link Technologies. Phosphoramidites for 2′-aminoethoxy-5-(3-aminoprop-1-ynyl)uridine (BAU) [21,22], 5-(1-propargylamino)-2′-deoxyuridine (U^P) [15], 2-aminopyridine (P) [26,28] and 3-methyl-2-aminopyridine (^{Me}P) [25,28] were prepared as previously described. The oligonucleo-

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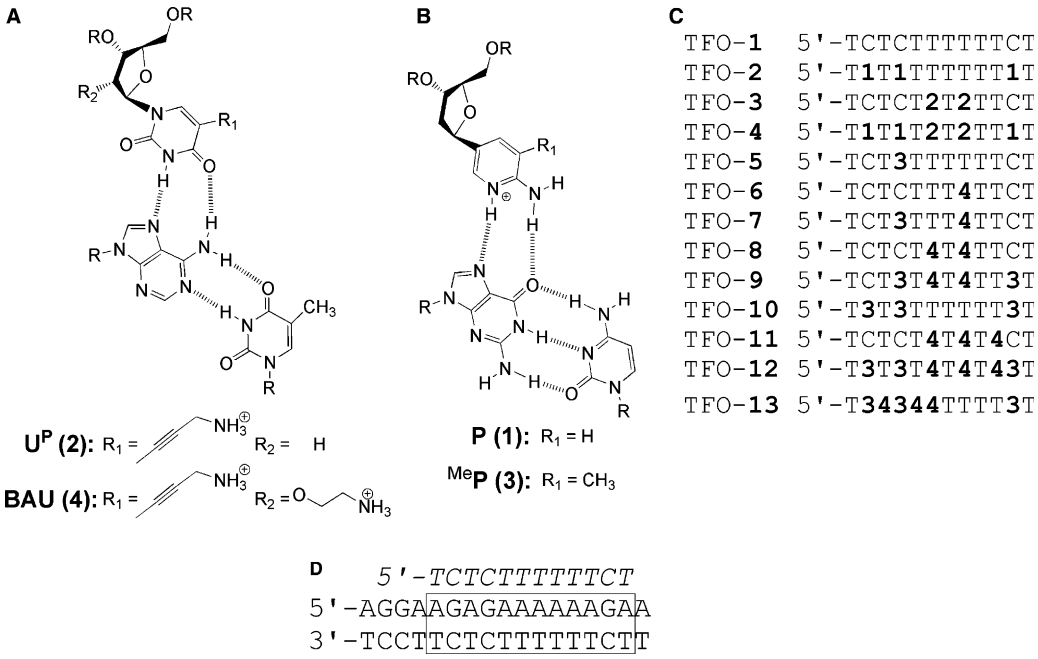

Fig. 1. (A) Chemical structures of 5-(1-propargylamino)-2′-deoxyuridine (U^P, **2**) and 2′-aminoethoxy-5-(3-aminoprop-1-ynyl)uridine (BAU, **4**) and the triplets that these form with an AT base pair. (B) Chemical structures of 2-aminopyridine (P, **1**) and 3-methyl-2-aminopyridine (^{Me}P, **3**) and triplets that these form with a GC base pair. (C) Sequences of the 12-mer oligonucleotides used in this work. (D) Sequence of the 17-base pair oligopurine tract in *tyr*T(43–59); the 12 base pair region targeted by the oligonucleotides shown in (C) is boxed and the third strand oligonucleotide (TFO-1) is shown above.

tides were deprotected for 24 h in 2 ml of 30% aqueous methylamine in the presence of phenol (5 mg). The deprotected oligonucleotides were purified by reversed-phase HPLC on a Brownlee Aquapore column (C8) using a gradient of acetonitrile in 0.1 M ammonium acetate. Purified oligonucleotides were analysed by MALDI-TOF MS using a ThermoBioAnalysis Dynamo MALDI-TOF mass spectrometer in positive ion mode using internal T_n standards [34]. The sequences of the TFOs used in this work are shown in Fig. 1C.

2.2. DNA fragments

The *tyr*T(43–59) fragment contains a 17-base oligopurine tract between positions 43 and 59 [35]; we have targeted a 12 base pair region within this tract with the TFOs as shown in Fig. 1D. A 110 base pair radiolabelled fragment containing this sequence was obtained by digesting the plasmid with *Eco*RI and *Ava*I and labelling at the 3′-end of the *Eco*RI site using reverse transcriptase and [α-^{32}P]dATP. This was then separated from the remainder of the plasmid DNA on an 8% (w/v) non-denaturing polyacrylamide gel. After elution the fragment was dissolved in 10 mM Tris–HCl, pH 7.5, containing 0.1 mM EDTA to give about 10 cps/μl as determined on a hand held Geiger counter (<10 nM).

2.3. DNase I footprinting

DNase I footprinting was performed by mixing radiolabelled DNA (1.5 μl) with the triplex-forming oligonucleotide (3 μl) dissolved in the appropriate buffer. Experiments at pH 5.0 were performed in 50 mM sodium acetate, at pH 6.0 in 10 mM PIPES containing 50 mM NaCl and at pH 7.0 and 7.5 in 10 mM Tris–HCl containing 50 mM NaCl. In some instances when no binding was observed with 30 μM TFO at pH 5, the buffers were supplemented with 2.5 mM $MgCl_2$. The final oligonucleotide concentrations varied between 1 nM and 30 μM. The complexes were left to equilibrate at 20 °C overnight. DNase I digestion was carried out by adding 2 μl of DNase I (typically 0.01 U/ml) dissolved in 20 mM NaCl containing 2 mM $MgCl_2$ and 2 mM $MnCl_2$. The reaction was stopped after 1 min by adding 4 μl of 80% formamide containing 10 mM EDTA, 10 mM NaOH, and 0.1% (w/v) bromophenol blue.

2.4. Gel electrophoresis

The products of digestion were separated on 10% polyacrylamide gels containing 8 M urea. Samples were heated to 100 °C for 3 min, before rapidly cooling on ice and loading onto the gel. Polyacrylamide gels (40 cm long, 0.3 mm thick) were run at 1500 V for about 2 h and then fixed in 10% (v/v) acetic acid. These were transferred to Whatman 3MM paper and dried under vacuum at 86 °C for 1 h. The dried gels were subjected to phosphorimaging using a Molecular Dynamics Storm phosphorimager.

2.5. Quantitative analysis

The intensity of bands within each footprint was estimated using ImageQuant software. These intensities were then normalized relative to a band in the digest which is not part of the triplex target site, and which was not affected by addition of the oligonucleotides. Footprinting plots [36] were constructed from these data and fitted using simple binding curves using Sigmaplot for Windows. C_{50} values, indicating the TFO concentration that reduces the band intensity by 50%, were calculated from these.

3. Results

We have previously shown that propargylamino-dU (U^P) [15] and bis-amino-U (BAU) [21,22] have enhanced affinity for AT base pairs compared with T and we and others have shown that 2-aminopyridine enables recognition of GC pairs at higher pHs than C [26–28]. We have now examined how these nucleoside analogues can be used in combination to achieve high affinity recognition of oligopurine tracts at physiological pH. For these studies, we have examined triplex formation at a 12 base oligopurine tract located within *tyr*T(43–59) (Fig. 1D). Since this target contains three GC pairs triplex formation with oligonucleotides containing only natural bases is

strongly pH dependent, while the nine AT base pairs result in a complex with relatively low affinity.

Fig. 2 shows representative footprinting patterns at this target site obtained at pH 5.0 with three different oligonucleotides, while similar experiments at pH 7.0 are shown in Fig. 3. The C_{50} values obtained for all 12 differently modified derivatives at four pHs are presented in Table 1. The unmodified oligonucleotide (TFO-1) shows no interaction with this target site (Fig. 2, panel 1) in 50 mM sodium acetate pH 5.0, but binds well on addition of 2.5 mM $MgCl_2$. As expected this interaction is strongly pH dependent and no footprint was observed at pH 6.0. Replacing all three of the cytosines with 2-aminopyridine (**1**, P) (TFO-2) causes a reduction in affinity at pH 5.0, though footprints are still observed at pH 6.0. A similar effect is seen with three substitutions of 3-methyl-2-aminopyridine (**3**, ^{Me}P) (TFO-10). Again both these oligonucleotides require magnesium for triplex formation. Substitution of only one C with ^{Me}P (TFO-5) causes a slight reduction in affinity at pH 5.0 relative to the unmodified oligonucleotide and has little effect on the pH dependency. These results suggest that each substitution of P or ^{Me}P for C causes a slight reduction in the affinity at pH 5.0, and extends triplex formation in the presence of magnesium to pH 6.0.

In TFO-3 two of the third strand Ts are replaced with U^P (**2**). This has little effect on either the affinity or pH dependence, as expected since our previous studies [15] have shown that several substitutions with this base analogue are required to improve triplex affinity. However, a single substitution with bis-amino-U (**4**, BAU) (TFO-6) generates a complex which, although not strong, is stable in the absence of magnesium. A further enhancement in affinity is seen with two substitutions of BAU (TFO-8), producing a complex which is also stable at pH 6.0. TFO-11 has a third substitution with BAU and this further enhances triplex stability at pH 5.0 and 6.0, though there is still no evidence of triplex formation at pH 7.0.

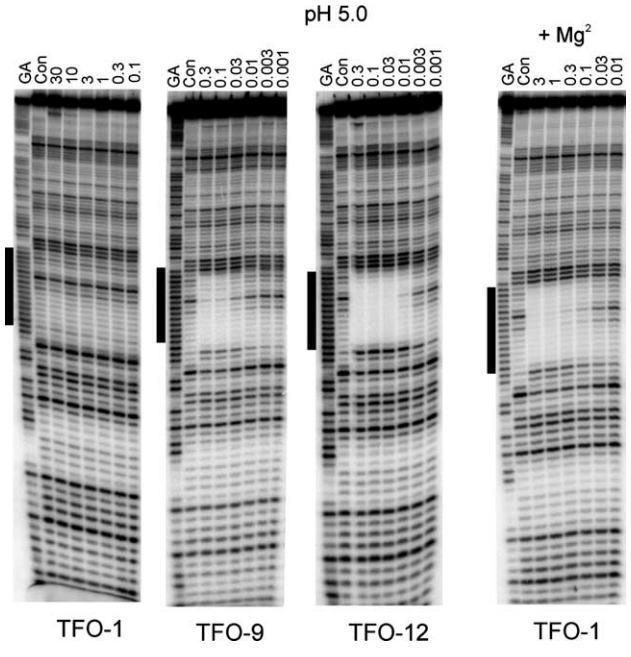

Fig. 2. DNase I footprints for the interaction of TFO-1, TFO-9 and TFO-12 with the *tyr*T(43–59) DNA fragment. The 12 base pair target site is indicated by the filled boxes. Oligonucleotide concentrations (μM) are shown at the top of each gel lane. The reactions were all performed in 50 mM sodium acetate pH 5.0, except for the final panel with TFO-1, for which 2.5 mM $MgCl_2$ was added to the buffer. Tracks labelled "GA" are sequencing lanes that are specific for purines (G+A).

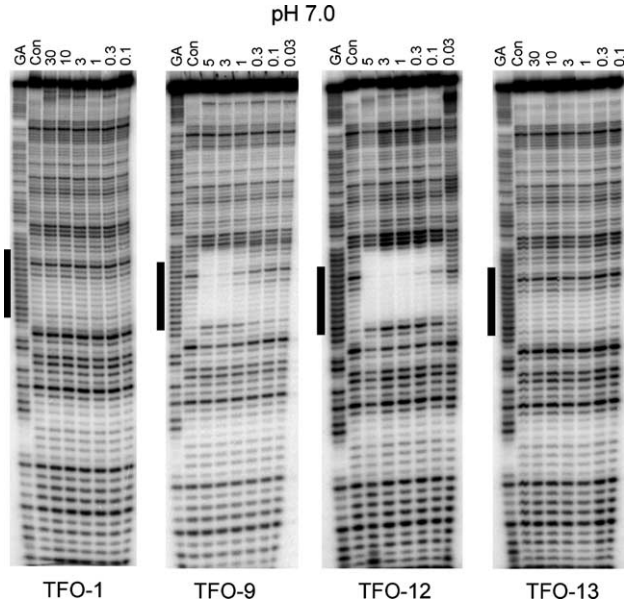

Fig. 3. DNase I footprints for the interaction of TFO-1, TFO-9, TFO-12 and TFO-13 with the *tyr*T(43–59) DNA fragment. The 12 base pair target site is indicated by the filled boxes. Oligonucleotide concentrations (μM) are shown at the top of each gel lane. The reactions were all performed in 10 mM Tris–HCl, pH 7.0, containing 50 mM NaCl. Tracks labelled "GA" are sequencing lanes that are specific for purines (G+A).

The remaining oligonucleotides contain substitutions for both T and C. TFO-4 contains two substitutions with P and two with U^P; this generates complex which is still dependent on the presence of magnesium and has enhanced affinity relative to TFO-2, and better pH stability than TFO-3. TFO-7, with one BAU and one ^{Me}P has a similar affinity and pH dependency to TFO-6. However, addition of two of each of these residues (TFO-9) enhances the affinity at pH 6.0, and generates a complex which now produces a footprint at pH 7.0 (Fig. 3, panel 2). TFO-12 contains three substitutions with ^{Me}P and three with BAU and this oligonucleotide generates a triplex with enhanced affinity at pH 5.0 (Fig. 2, panel 3), which is also stable at pH 7.0 (Fig. 3, panel 3) and still binds with a C_{50} value of 200 nM at pH 7.5. This oligonucleotide produces the strongest complex which extends to a higher pH than all the others. TFO-13 has the same base composition as TFO-12, with three ^{Me}P and three BAU residues, but in this instance the modifications are placed close together towards the 5′-end of the oligonucleotide, rather than being distributed throughout the third strand. This complex is stable at pH 5.0 in the absence of magnesium, and so clearly binds better than the unmodified oligonucleotide TFO-1. It also produces a stable complex at pH 6.0, though this is not as stable as that produced by TFO-9 and TFO-12. However, this oligonucleotide failed to produce a footprint at pH 7.0 and above, in contrast to TFO-12, suggesting that the arrangement of modified residues is important and that distribution, rather than clustering, of positively charged residues produces the most stable complexes.

Table 1
C_{50} values for the interaction of the various modified 12-mer oligonucleotides with the oligopurine target site in *tyr*T(43–59)

TFO	pH 5.0 (nM)	pH 6.0 (nM)	pH 7.0 (nM)	pH 7.5 (nM)
TFO-1[a]	24 ± 2	n.d.	–	–
TFO-2[a]	300 ± 100	6300 ± 1000	n.d.	–
TFO-3[a]	22 ± 2	n.d.	–	–
TFO-4[a]	15 ± 2	3800 ± 500	n.d.	–
TFO-5[a]	50 ± 8	n.d.	–	–
TFO-6	400 ± 200	n.d.	–	–
TFO-7	500 ± 300	n.d.	–	–
TFO-8	29 ± 10	6900 ± 1400	n.d.	–
TFO-9	39 ± 8	200 ± 100	600 ± 100	n.d.
TFO-10[a]	200 ± 100	3200 ± 1000	n.d.	–
TFO-11	8.3 ± 1.8	21 ± 2	n.d.	–
TFO-12	2.8 ± 0.8	8 ± 5	35 ± 4	200 ± 100
TFO-13	40 ± 7	400 ± 100	n.d.	–

n.d. = no footprint detected at a third strand concentration of 30 μM.
[a]2.5 mM $MgCl_2$ was added, as no footprint was observed at 30 μM TFO at pH 5.0 in its absence.

4. Discussion

The results presented in this paper demonstrate that oligonucleotides that contain both bis-amino-U (**4**, BAU) and 3-methyl-2-aminopyridine (**3**, ^{Me}P) can be used to enhance triplex stability and to generate complexes that are stable at physiological pH. We have previously used these nucleosides, in combination with other analogues, to achieve recognition of all four base pairs by triple helix formation at physiological pH [37] though these complexes with 19-mer oligonucleotide required micromolar concentrations of the third strand to generate a footprint at pH 7.0 as a result of the presence TA and CG interruptions within the target duplex. These two nucleosides seem to operate in a cooperative fashion to stabilize triplex formation at higher pHs. Oligonucleotides that contain only one of these modified bases showed enhanced affinity at pH 6.0, but only the combination produced complexes that were stable at pH 7.0 and above.

When used alone, 2-aminopyridine and its 3-methyl derivative increase the pH range for forming triplexes. However, at low pH these triplexes are less stable than those with T and C. This reduction in affinity is reversed by combining this cytosine analogue with BAU. In addition, oligonucleotides that contain analogues P, ^{Me}P and U^P all require magnesium to form stable triplexes, while bis-amino-U (**4**) removes the requirement for this divalent cation.

These results demonstrate that the cooperative enhancement in triple stability obtained with these two analogues depends on the sequence arrangement of the third strand. Their effect is much greater when they are evenly distributed throughout the oligonucleotide instead of being clustered at one end. This contrasts with studies using 2′-aminoethoxy modified oligonucleotides, which suggested a greater effect when the modifications were clustered together [38], though these experiments were performed with psoralen-linked oligonucleotides. However, the greater affinity obtained when there is an alternation of positive and neutral nucleosides is similar to that seen with third strand using T and C for which the most stable triplexes are obtained with alternating $C^+ \cdot GC$ and $T \cdot AT$ triplets [14,39].

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